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Physiologic aging of mature porcine erythrocytes: Effects of various metabolites, antimetabolites, and physical stressors

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SUMMARY

Red blood cells were collected from Landrace × Duroc pigs in pooled and single batches. The RBC were stored for 24 hours to 20 days and exposed to 1 or more chemical and physical stressors. The chemicals were pyruvate, lactate, inosine, concanavalin A-luminol-bovine serum albumin conjugate, hydrogen peroxide, L-mimosine, and 3-amino-L-tyrosine. Physical stressors included thermogenic microwave radiation (2,450 MHz, mean specific absorption rate of 91 W/kg) and conventional heating with hot air or hot-water bath. Heating erythrocytes to 43 C for 10 minutes with microwaves or hot air did not significantly ($P > 0.05$) increase hemolysis, compared with hemolysis of RBC at 4 C (controls). Pyruvate or lactate did not affect the RBC under these conditions. Hemolysis of cells coated with concanavalin A-luminol-bovine serum albumin and heated to 43 C for 10 minutes by use of microwaves or hot air was significantly ($P < 0.05$ by Student's t test) greater (48%) than that of RBC at 4 C (controls). The method of heating or the presence of pyruvate, lactate, or inosine did not have a significant ($P > 0.05$) effect on hemolysis. Lysis of RBC (14 days after collection) coated with concanavalin A-luminol-bovine serum albumin and stored at 4 C was not significantly different ($P > 0.05$) than that of noncoated cells (6 days after collection) stored at 4 C (control). When RBC were heated 20 days after collection to 48 C for 30 minutes, using a hot water bath, hemolysis was 60.6% greater than that of control cells (4 C). The L-mimosine (340 μM) did not affect thermal hemolysis. The 3-amino-L-tyrosine (74 μM) suppressed thermal hemolysis to that of the controls. When RBC were treated 19 days after collection with 44 mM H_2O_2 at 37 C for 15 minutes, their binding capacity for luminol-bovine serum albumin was 721% more than that of control cells at 37 C. In a second single batch of RBC, the increase in binding induced by H_2O_2 (11.7 mM) was inhibited 51% by 1.49 mM 3-amino-L-tyrosine. Red blood cells collected 72 hours before use and preincubated with pyruvate (1.33 mM) for 30 minutes at 37 C (hot air) had 2.66 times greater luminol-bovine serum albumin binding than did controls (37 C). Preincubation with 1.49 mM 3-amino-L-tyrosine totally inhibited the increase in binding with pyruvate. Therefore, the effectiveness of 3-amino-L-tyrosine for protecting natural membrane components from oxidation is related to its thermoprotective properties. The luminol-bovine serum albumin binding assay was a sensitive, simple technique for detecting oxidative physiologic aging of porcine RBC.

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Metabolism of mature erythrocytes controls and is affected by physiologic aging of the RBC. The physiologic aging of erythrocytes is involved in the pathogenesis of anemia due to inflammation and is important in the clinical effectiveness of blood transfusions.[1-3] Cellular physiologic aging is defined as the structural and functional alterations of the cell membrane as a result of chemical (eg, halogenated hydrocarbons, phenylhydrazine), biochemical (eg, endotoxin, endogenous pyrogens), or physical (eg, hyperthermia) metabolic stressors that result in a shortened circulating half-life.[1-3] Furthermore, inborn deficiencies in antioxidative biochemical pathways increase the inherent sensitivity of erythrocytes to membrane oxidation (physiologic aging) induced by chemical and physical metabolic stressors.[4]

Methods for determining membrane oxidation associated with physiologic aging of erythrocytes include determination of the malondialdehyde content of membranes and determination by fluorescence of oxidation products that accumulate within cell membranes.[5] These techniques involve organic extractions of membrane components and spectrophotometric or spectrofluorometric techniques, respectively. Artifacts such as autoxidation of unsaturated fatty acid acyl groups or dissociation of reversibly cross-linked membrane protein may result due to manipulation required in processing the erythrocytes.

Purposes of the present study were to evaluate thermal cellular fragility and various chemiluminescent techniques as diagnostic and prognostic methods for determining oxidative physiologic aging of erythrocytes, and (using these methods) to determine the effects of various metabolites, antimetabolites, and physical stressors on autoxidation, physiologic aging, of porcine RBC.

Materials and Methods

Source of RBC—Blood samples were collected from 4 Landrace × Duroc pigs in a closed herd. Pigs in the herd previously had developed porcine stress syndrome (malignant hyperthermia) after halothane-induced anesthesia. Blood samples were collected into acidic citrated dextrose solution in standard sterile evacuated glass tubes (7 ml).[a] Samples (1-ml aliquots) were

[a] Vacutainer, Becton, Dickinson, & Co, Rutherford, NJ

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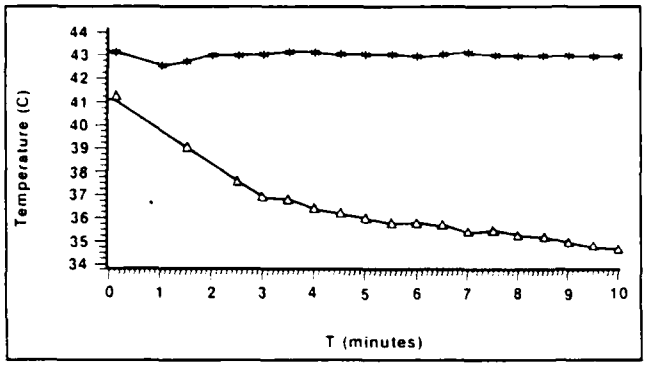

Fig 1—Temperature of RBC (*) and of the air chamber (△) enclosing the RBC during microwave (2,450 MHz) irradiation (the mean specific absorption rate was 91 ± 4 W/kg).

TABLE 1—Thermal hemolysis of porcine RBC 6 days after collection

	43 C for 10 minutes					
	Microwave exposed		Hot air		Controls (RBC stored at 4 C)	
Reagents added	Hemolysis* (mean ± SD)	(n)	Hemolysis* (mean ± SD)	(n)	Hemolysis* (mean ± SD)	(n)
None	0.387 ± 0.042	5	0.356 ± 0.059	3	0.371 ± 0.060	4
Pyruvate (10 m*M*)	0.374 ± 0.055	4	0.427 ± 0.015	4	0.363	1
Lactate (10 m*M*)	0.424 ± 0.014	4	0.440 ± 0.078	4	0.362	1

* Optical absorbance at 410 nm of diluted supernatant (1:2). Significant differences were not found between any 2 means by use of the Student's *t* test ($P > 0.05$).

(n) = No. of replicates.

diluted 1:10 in phosphate (0.1*M*)-buffered saline solution (PBSS; pH 7.4), and centrifuged at 200 × g for 20 minutes. Erythrocytes were suspended in fresh PBSS, counted by use of an automated cell counter,[b] and stored at 4 C until assayed.

Heating—Red blood cells were heated in 1- or 1.5-ml aliquots of PBSS pH 7.4, with or without additional reagents, in cellulose nitrate or polypropylene centrifuge tubes. Two concentrations of cells were used: 9×10^8 and 1.4×10^9 cells/ml. Heating was accomplished by use of microwave radiation, hot air, or a hot water bath. Microwave heating[c] of the sample at a mean (± SD) specific absorption rate of 91 ± 4 W/kg (2,450 MHz) was offset by an air-cooling system that maintained the sample at a constant temperature (± 0.2 C, Fig 1).[d] In the absence of microwave radiation, the temperature was held constant by hot air alone. The microwave-hot air system was maintained at 37 C for 15 minutes and then the temperature was increased to 43 C by use of microwaves or hot air. Hot water bath heating maintained the temperature within ± 0.5 C. Temperature was maintained at 37 C for 15 or 30 minutes, 43 C for 10 minutes, 45 C for 30 minutes, and 48 C for 30 minutes, regardless of the heating technique used.

Thermal fragility—Hemoglobin released into the supernatants of the heated cells was determined as described.[e] Briefly, 1.5-ml suspensions of heated cells were centrifuged (13,400 × g for 1 minute); the supernatant was collected and diluted 1:2 in PBSS (pH 7.4), and optical absorbances at 410 nm were determined by use of a dual-beam spectrophotometer.[f] The PBSS containing pyruvate, lactate, inosine, hydrogen peroxide, L-mimosine, and/or 3-amino-L-tyrosine was used in some instances in place of PBSS to make the 1.5-ml RBC suspensions for thermal fragility experiments (Tables 1, 2, and 3).

[b] Model ZBI, Coulter Electronics Inc, Hialeah, Fla
[c] Model 10704, MCL Inc, La Grange, Ill
[d] Spectronic 2000, Bausch & Lomb, Rochester, NY

TABLE 2—Thermal hemolysis of concanavalin A-luminol-bovine serum albumin-coated porcine RBC 11 to 14 days after collection

	43 C for 10 minutes					
	Microwave exposed		Hot air		Controls (RBC stored at 4 C)	
Reagents added	Hemolysis* (mean ± SD)	(n)	Hemolysis* (mean ± SD)	(n)	Hemolysis* (mean ± SD)	(n)
None	0.617 ± 0.03	4	0.632 ± 0.047	4	0.426 ± 0.027†	3
Pyruvate (10 m*M*)	0.646 ± 0.07	4	0.646 ± 0.02	4	NA	NA
Lactate (10 m*M*)	0.643 ± 0.02	4	0.667 ± 0.04	4	NA	NA
Inosine (10 m*M*)	0.578 ± 0.045	4	0.597 ± 0.080	4	NA	NA

* Optical absorbance at 410 nm of diluted supernatants. † Significantly different ($P < 0.05$; Student's *t* test) from all other means in the table.
(n) = No. of replicates. NA = not applicable.

TABLE 3—Thermal hemolysis of porcine RBC heated to 48 C for 30 minutes 20 days after collection

	Hemolysis* (410 nm absorbance)		Percentage difference from the corrected *control* value	
Reagents added	Actual (mean ± SD)	Corrected	48 C	4 C
None (4 C control)	0.371 ± 0.039	0.371	−37.8†	NA
None (48 C control)	0.795 ± 0.029	0.596	NA	60.6†
L-mimosine (340 μ*M*)	0.965 ± 0.026	0.724	21.5†	95.1†
3-amino-L-tyrosine (74 μ*M*)	0.491 ± 0.032	0.368	−38.3†	−0.8

* Performed in triplicate and corrected for the difference in RBC dilution from the 4 C control. † Corrected means are significantly different from the control mean ($P < 0.05$); Student's *t* test.
NA = not applicable.

Preparation of concanavalin A-luminol-bovine serum albumin (conA-lu-BSA) conjugate—The lu-BSA complex was made by mixing 2 mg of luminol (5-amino-2,3-dihydrophthalazine-1,4-dione), 0.2 ml of 2 mg of BSA/ml of PBSS (pH 6.9), and 1.6 ml of PBSS (pH 6.9). The preparation was then passed through a 0.22-μm nitrocellulose filter to remove insoluble luminol. Next, 0.2 ml of 2 mg of conA/ml of PBSS (pH 6.9) was added to the filtrate. After gentle mixing, 10 μl of 25% glutaraldehyde was added. The solution was incubated in the refrigerator for 1 hour. To stop conjugation, 100 mg of glycine was added to the solution. The solution was then passed through a 48-ml column of dextran gel-filtration beads equilibrated with PBSS (pH 6.9). The gel exclusion size was ≥ 5,000 molecular ratio for globular molecules. Four-milliliter fractions were collected from the gel-filtration column and evaluated for luminescent-labeling activity.

Determining RBC surface peroxidation with conA-lu-BSA—One milliliter of supernatant from each 10-ml aliquot of 1:20 diluted RBC was replaced with 1 ml of conA-lu-BSA as previously described. The preparation was incubated for 15 minutes at 37 C in a water-jacketed cell culture incubator.[g] Then, the cells were centrifuged (13,400 × g for 1 minute), suspended in PBSS, and washed in the same manner 2 additional times. The cells were maintained at 4 C until used the same day of preparation.

After exposure to chemical or physical stressors, 100 μl of the RBC suspension was added to 500 μl of PBSS (pH 7.4) containing 0.3% hydrogen peroxide in a scintillation vial. To activate the sample, 100 μl of 0.1*N* NaOH was added to the vial and the vial was placed in a photometer.[h] The photometer was set to a dark count of 1 count 30 s at a sensitivity of 600 arbitrary instrument units at room temperature (approx 23 C). All counts were made after a 5-s delay to allow for stabilization of the photomultiplier. The counts were reported as counts per 30 s.

Determination of lu-BSA binding—Porcine RBC were treated with hydrogen peroxide, pyruvate, and or 3-amino-L-tyrosine at 4, 37, or 45 C. Exposure times were 15 minutes, 30 minutes, or

[e] Model 3126, Forma Scientific, Division of Mallinckrodt Inc, Marietta, Ohio
[f] Integrating ATP Photometer, model 3000, Science Applications Inc, San Diego, Calif

TABLE 4—Chemiluminescence (CL) of concanavalin A-luminol-bovine serum albumin-labeled porcine RBC in H_2O_2 base system* 11 to 18 days after collection

Reagents added	CL (counts/30 s; mean ± SD)	(n)	Percentage of control value
None (control, labeled)	30,319 ± 1,201	6	NA
None (no cells)	62 ± 7‡	3	0.2
pyruvate (1.43 mM)	420‡	1	1.4
RBC (nonlabeled)	13,351 ± 2,119‡	4	44
Pyruvate (1.43 mM)	60,732 ± 3,258‡	4	200
Lactate (1.43 mM)	30,930 ± 3,399	5	102
Inosine (1.43 mM)	34,972 ± 2,732‡	4	115
L-mimosine (630 μM)	8,555 ± 1,044‡	3	28
pretreatment (5 mM)†	28,517 ± 2,591	3	94
3-Amino-L-tyrosine			
(1.44 mM)	844 ± 165‡	4	3
(160 μM)	5,430 ± 1,266‡	3	18
pretreatment (11.5 mM)†	22,457 ± 2,128‡	3	74
None (48 C, 30 min)	27,637 ± 1,162‡	3	91

* 100 μl of a 1:30 dilution of RBC activated by addition of 500 μl of 0.3% H_2O_2 in phosphate-buffered saline solution (pH 7.4) and 100 μl of 0.1N NaOH. † Cells were incubated for 30 minutes and washed before activation. ‡ Means are significantly different ($P < 0.05$; Dunnett's test) from control value.

(n) = No. of replicates. NA = not applicable.

TABLE 5—Base-activated chemiluminescence (CL) of luminol-bovine serum albumin bound to porcine RBC*

Reagent pretreatment	Temperature for 30 minutes of pretreatment	CL† Counts/30 s (mean ± SD)	(n)	Percentage difference from control value
None (control)	4 C	380 ± 63	4	NA
None	37 C	583 ± 78	4	53
H_2O_2 (11.7 mM)	37 C	1,346 ± 386	4	254
3-Amino-L-tyrosine (1.49 mM) and H_2O_2 (11.7 mM)	37 C	663 ± 145	4	74
None	45 C	600 ± 110	4	58
Pyruvate (1.33 mM)	45 C	1,203 ± 266	3	216
3-Amino-L-tyrosine (1.49 mM)	45 C	550 ± 126	4	45

* 100 μl of a 1:30 dilution of RBC < 24 hours after collection that had been labeled by overnight incubation with luminol-bovine serum albumin at 4 C. † Cells without label had a mean (± SD) 5 ± 3 counts/30 s when base activated.

(n) = No. of replicates. NA = not applicable.

TABLE 6—Base-activated chemiluminescence (CL) of porcine RBC labeled with luminol-bovine serum albumin at 37 C*

Reagent pretreatment (37 C for 30 min)	CL Counts/30 s (mean ± SD)	(n)	Percentage difference from control value
None (control)	883 ± 129	3	NA
3-Amino-L-tyrosine (1.49 mM)†	794 ± 156	4	−10
Pyruvate (1.33 mM)	2,348 ± 646	3	166
3-Amino-L-tyrosine (1.49 mM) and pyruvate (1.33 mM)	964 ± 230	3	9

* 100 μl of a 1:30 dilution of RBC 72 hours after collection that had been labeled by use of a 30-minute incubation at 37 C. † Preincubated at 4 C for 72 hours.

(n) = No. of replicates. NA = not applicable.

72 hours. Reagents were removed by centrifugation. Three binding methods were used: 0.25 ml of 1:20 RBC were incubated with 0.25 ml of lu-BSA (1 mg/ml) in PBSS (pH 6.9) for 30 minutes at 37 C (method 1); 0.05 ml of packed RBC was suspended in 0.2 ml of the lu-BSA solution and incubated overnight at 4 C (method 2; Table 5); and 0.05 ml of packed RBC was suspended in 0.2 ml of lu-BSA solution and incubated at 37 C for 30 minutes (method 3; Table 6). Cells in the 3 methods were washed once in PBSS (pH 7.4) and suspended in PBSS to a final dilution of 1:20 (method 1) or 1:30 (methods 2 and 3), then 100 μl of each cell suspension was added to 500 μl of PBSS (pH 7.4) in a scintillation vial. The vial was placed in the photometer and the solution was activated by addition of 100 μl of 0.1N NaOH. Settings for the photometer were as previously described for the conA-lu-BSA.

Results

Thermal hemolysis—The concentration of blood cells was adjusted to 9×10^8/ml for Tables 1 and 2; 1.4×10^9/ml for Table 3; and 9×10^8/ml for Tables 4, 5, and 6. Data (Table 3) were corrected for RBC concentration for comparison with control data (Table 1). Six days after collection porcine RBC had insignificant ($P > 0.05$; Student's t test) increases in hemolysis at 43 C in PBSS (pH 7.4) or PBSS containing 10 mM lactate or pyruvate when compared with controls in PBSS at 4 C (Table 1). A distinction was not found between microwave and hot-air heating effects.

Labeling porcine RBC 11 to 14 days after collection with conA-lu-BSA sensitized the cells to thermal hemolysis (43 C for 10 minutes; Table 2). Comparison of 4 C controls (ie, cells stored at 4 C; Tables 1 and 2) did not indicate a significant change in thermal fragility during the 6- to 14-day postcollection period and indicated that conA-lu-BSA-coated cells were no more fragile than were uncoated cells at 4 C. Storing porcine RBC in inosine (10 mM) did not significantly reduce thermal fragility when compared with samples heated to 43 C for 10 minutes in PBSS (pH 7.4) alone.

Cells incubated 20 days after collection at 48 C for 30 minutes in PBSS released 60.6% more hemoglobin than did the 4 C control (Table 3). Mimosine at a final concentration of 340 μM significantly ($P < 0.05$, Student's t test) increased thermal hemolysis. At a final concentration of 74 μM, 3-amino-L-tyrosine suppressed thermal hemolysis to the level of the 4 C control.

ConA-lu-BSA chemiluminescence—Chemiluminescence (CL) of conA-lu-BSA-labeled porcine RBC (6×10^7 cells/sample) developed when mixed with 63 mM exogenous H_2O_2 and 0.014N NaOH (Table 4). The cells were mixed with a variety of metabolites and antimetabolites before activation of the CL by addition of the H_2O_2 and NaOH. The postcollection age of these cells was 11 to 18 days and the cells were assayed on the day of labeling. Nonlabeled porcine RBC had a large amount of natural CL when mixed with H_2O_2 and NaOH (56% less than labeled RBC). Pyruvate (1.43 mM), an autoxidizable substrate, doubled the CL of labeled cells when compared with the CL of labeled cells in PBSS without additional chemicals (positive control). Lactate (1.43 mM) induced an insignificant ($P > 0.05$) increase in CL when compared with the positive control. Inosine induced a small, but significant ($P < 0.05$), increase in labeled-cell CL in comparison with the positive control. A small (9%), but significant ($P < 0.05$), decrease in the residual CL, compared with CL of unheated samples of labeled RBC in PBSS, was induced when samples were heated to 48 C in a water bath for 30 minutes. At 630 μM, L-mimosine inhibited the CL of labeled RBC, when compared with samples in PBSS without L-mimosine, by 72%, which was 36% below natural RBC CL. Prewashing of labeled cells for 30 minutes at room temperature in 5-mM L-mimosine did not significantly effect CL when compared with that of nontreated labeled porcine RBC. Therefore, little if any mimosine entered or

adhered to luminescent sites on the cells. 3-Amino-L-tyrosine (1.44 m*M*) inhibited CL of labeled RBC by 97%, which was 94% below natural CL. At 160 μ*M*, 3-amino-L-tyrosine inhibited CL of labeled RBC by 82%. Some 3-amino-L-tyrosine was absorbed when conA-lu-BSA-coated porcine RBC were washed 30 minutes at room temperature in PBSS containing 11.5-m*M* 3-amino-L-tyrosine. After removal of the 3-amino-L-tyrosine from the labeled RBC by centrifuging and suspending the cells in PBSS, activation of these cells with peroxide and base induced 26% less CL than that of nontreated labeled RBC in PBSS.

Lu-BSA chemiluminescence—Exposure of 1.4 × 10^9 RBC/ml to 44 m*M* H_2O_2 for 15 minutes at 37 C (hot air heating) resulted in CL of 558 ± 157 counts/30 s (n = 4) after nonspecific binding of lu-BSA (method 1) and activation with 0.1*N* NaOH. This binding of lu-BSA after H_2O_2 treatment was 721% more (as determined by base-activated CL) than for cells maintained in PBSS (pH 7.4) for 15 minutes at 37 C. The mean CL for the nontreated cells was 68 ± 22 counts/30 s (n = 4). The postcollection age of the RBC assayed by method 1 was 19 days. The natural CL of nonlabeled cells treated with base was 5 counts/30 s (Table 5).

Effects of 3-amino-L-tyrosine on lu-BSA chemiluminescence and hemolysis—When RBC were incubated overnight at 4 C, 3-amino-L-tyrosine inhibited the effects of hydrogen peroxide at 37 C on the nonspecific binding and CL of lu-BSA (Table 5). The RBC binding of lu-BSA induced by H_2O_2 (11.7 m*M*) was decreased 51% by adding 1.49 m*M* 3-amino-L-tyrosine to the peroxide solution. The CL of cells treated with H_2O_2 and 3-amino-L-tyrosine solution was 14% greater than the 37 C control value.

Heating RBC to 45 C (without additional reagents) for 30 minutes (hot-water bath) bound 58% more lu-BSA than the 4 C control cells, but only 3% more than the 37 C control. Heating RBC to 45 C in pyruvate (1.33 m*M*) bound 101% more lu-BSA than when RBC were heated to 45 C alone (without pyruvate).

Addition of 3-amino-L-tyrosine (1.49 m*M*) did not significantly inhibit thermal hemolysis at 45 C. The mean (± SD) hemolysis of the 27 samples (Table 5) was 0.251 ± 0.01 (as determined by the optical density of the sample supernatants [1:2 dilution] at 410 nm). When RBC were treated with 3-amino-L-tyrosine and H_2O_2 at 37 C, hemolysis was significantly greater than that of the 4 C control (0.261 ± 0.007 vs 0.243 ± 0.004; $P < 0.005$, unpaired, 2-tailed Student's *t* test).

The RBC immediately stored at 4 C for 72 hours after collection in PBSS containing 1.49 m*M* 3-amino-L-tyrosine, and labeled by incubating lu-BSA with the RBC for 30 minutes at 37 C, bound 10% less lu-BSA than those stored in PBSS alone. The 3-amino-L-tyrosine was not replaced with fresh reagent during the 30-minute incubation at 37 C. Treatment of RBC with pyruvate (1.33 m*M*) for 30 minutes at 37 C increased the lu-BSA binding when compared with RBC in PBSS alone by 2.66 times. Cells that had been preincubated in 3-amino-L-tyrosine were protected against the effects of pyruvate. The mean hemolysis (optical absorbance of the supernatant of the cells at 410 nm) for the 37 C control was 0.340 for 2 samples. For samples treated with pyruvate and with pyruvate and 3-amino-L-tyrosine, the mean hemolysis at 37 C was

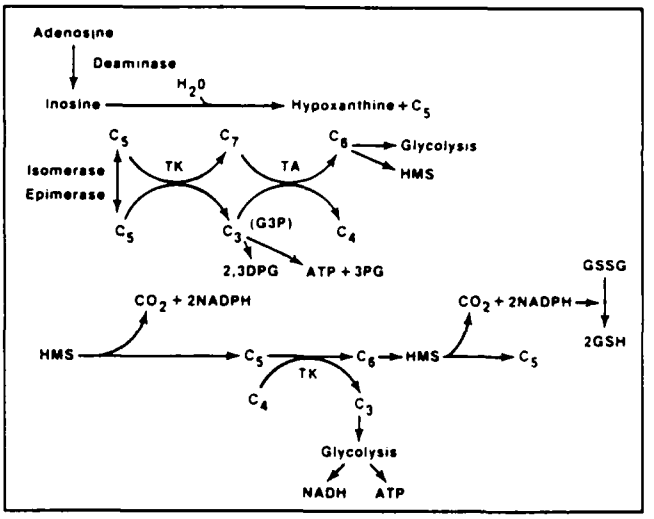

Fig 2—Proposed pentose phosphate pathways. C_5 = ribose; C_5' = xylulose; C_6 = glucose; G3P = glyceraldehyde-3-phosphate; C_4 = erythrose; C_7 = sedoheptulose; 2,3DPG = 2,3-diphosphoglycerate; 3PG = 3-phosphoglycerate; HMS = hexose monophosphate shunt; TK = transketolase; TA = transaldolase; GSSG = oxidized glutathione; and GSH = reduced glutathione. The C_5 sugars in the pathway may be phosphorylated.

0.362 ± 0.016 (n = 3) and 0.326 ± 0.03 (n = 3), respectively. Hemolysis of RBC preincubated with 3-amino-L-tyrosine alone was significantly ($P < 0.005$) higher than that of cells treated with pyruvate or 3-amino-L-tyrosine and pyruvate. The mean hemolysis of 3-amino-L-tyrosine treated cells without pyruvate was 0.452 ± 0.02 (n = 4).

Discussion

Physiologic aging of erythrocytes is controlled by the cellular nutritional state, storage temperature, inherent antioxidative defenses, exogenous chemical oxidants or inducers of autoxidation, and functioning carbohydrate catabolic pathways. Porcine RBC are unusual, in that the cells of some porcine breeds have inherent deficiencies in glutathione metabolism,[7] and RBC from pigs beyond the neonatal stage are unable to transport glucose.[8] Inosine is a probable source of endogenous glucose or of 3-carbon subunits derived via the pentose phosphate pathways (Fig 2). Porcine RBC have high endogenous concentrations of diphosphoglycerate and adenosine triphosphate.[8] Both metabolites are required for and produced by glycolysis in the RBC of other species.

The pentose phosphate pathways may produce glyceraldehyde 3-phosphate and nicotinamide-adenine dinucleotide phosphate (NADPH, Fig 2). The NADPH is a cofactor for glutathione reductase. Reduced glutathione (the product of this reductase) is used by glutathione peroxidase to reduce hydrogen peroxide and lipid peroxides that result from autoxidation of oxyhemoglobin.

Glyceraldehyde 3-phosphate can be broken down into pyruvate by glycolytic enzymes. This process yields 2 mol of adenosine triphosphate and 1 mol of nicotinamide adenine dinucleotide (NADH) per mole of glyceraldehyde 3-phosphate. Methemoglobin is reduced by the NADH via methemoglobin (cytochrome b_5) reductase to hemoglobin.[9] Through the action of aldolase, phosphatase, and

hexose phosphate isomerase, 2 mol of glyceraldehyde 3-phosphate also can be converted into glucose-6-phosphate. Glyceraldehyde 3-phosphate also can be converted to 3-phosphoglycerate by the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. The 3-phosphoglycerate and 1,3-phosphoglycerate are substrates for the production of 2,3-diphosphoglycerate by 2,3-diphosphoglycerate mutase.[9] Diphosphoglycerate is an important allosteric effector of oxygen binding by hemoglobin.[10]

The RBC of pigs prone to malignant hyperthermia (porcine stress syndrome) have greater thermo-CL than do RBC from resistant breeds of swine.[4] Thermo-CL depends on superoxide and hydrogen peroxide released from oxyhemoglobin during heating. Resistant cells can be made thermochemiluminescent by treating them with 1-chloro-2,4-dinitrobenzene, an agent that depletes cellular reduced glutathione.[4] Because heating increases the release of oxygen free radicals from erythrocytes (especially those from pigs prone to malignant hyperthermia[4]), one might expect the cells to be thermally fragile. Unfortunately, the relationship between thermal fragility and autoxidation is complex. In human RBC, treatment with 1-chloro-2,4-dinitrobenzene enhances hemolysis at 48 C, but decreases hemolysis at 42 C.[6] In the present study, the autoxidizable substrate pyruvate did not enhance thermal hemolysis at 43 C. This result appears to be in qualitative agreement with the human cell data.[6] Lactate, which can be metabolized by lactate dehydrogenase into NADH and pyruvate, did not affect thermal fragility at 43 C (Table 1).

The binding of conA-lu-BSA conjugate to porcine RBC increased thermal hemolysis at 43 C after a brief heating period (10 minutes), regardless of the heat source (microwave radiation or hot air). In other species, conA binds to Band 3 transmembrane protein.[11] This protein composes the anion channel and is associated with glucose transport.[11] The channel allows the passage of stable anions such as chloride and passage of superoxide.[12] Placing the conA-lu-BSA conjugate in close proximity to this channel maximized the chances of interaction between fluxing superoxide and bound luminol. Therefore, an autocatalytic cycle of luminol oxidation with cooxidation of membrane components may have been established. Enhanced thermal hemolysis of labeled RBC in the present study supports this hypothesis (Table 2). Pyruvate, lactate, and inosine did not influence hemolysis of labeled RBC at 43 C. The pyruvate and lactate data were in agreement with observations of nonlabeled cells. Inosine was considered a potential thermoprotectant because it is a potential metabolic source of NADPH via the hexose monophosphate shunt. The NADPH could maintain the reduced glutathione concentrations via glutathione reductase. The reduced glutathione could in turn provide reducing equivalent for the elimination of membrane damaging peroxides by glutathione peroxidase. Under the conditions of the present study, such protection was slight, if present at all. Therefore, this process was inoperable or ineffectual under the conditions of the present study.

The antimetabolites L-mimosine and 3-amino-L-tyrosine were considered potential RBC antioxidants because mimosine is a peroxidase and tyrosinase inhibitor[13,14] and because 3-amino-L-tyrosine is oxidizable and is a peroxidase inhibitor or alternate substrate.[15] Mimosine at 340 μM did not protect against thermal hemolysis at 48 C for 30 minutes (Table 3), whereas, 3-amino-L-tyrosine at a lower concentration (74 μM) was a powerful inhibitor. When hydrogen peroxide was added to RBC (9×10^7) labeled with conA-lu-BSA, high-intensity CL was produced (Table 4). This CL was inhibited by mimosine and 3-amino-L-tyrosine. As might be anticipated from the hemolysis data, 3-amino-L-tyrosine was a more effective inhibitor of CL than was mimosine. Pyruvate acted as a cooxidant, inosine slightly but significantly enhanced CL, and lactate was relatively inert in the CL reaction. At the appropriate concentrations, mimosine and 3-amino-L-tyrosine reduced the CL of RBC below the background intensity of peroxide-induced CL of natural membrane components. The effectiveness of 3-amino-L-tyrosine in protecting these natural membrane components from oxidation probably was related to its thermoprotective properties.

In experiments involving the nonspecific binding of lu-BSA to RBC (Tables 5 and 6), hydrogen peroxide and pyruvate increased labeling of RBC. Dialdehydes were probably formed via membrane oxidation. The aldehydes in turn may have bound the lu-BSA through aldimine (Schiff base[16]) formation. Exogenous dialdehyde (glutaraldehyde) binds lu-BSA to RBC membranes in such a way that its CL can be activated by addition of base without hydrogen peroxide.[4] Binding of 3-amino-L-tyrosine to the membrane via its α-amino group and subsequent inhibition of CL by inhibiting binding of lu-BSA probably did not occur because preincubation with 8 times the 3-amino-L-tyrosine used in the lu-BSA binding experiments induced only a 26% inhibition of CL (Table 4), indicating that little of the 3-amino-L-tyrosine was retained at the membrane surface. The subsequent washing removed loosely bound 3-amino-L-tyrosine. 3-Amino-L-tyrosine readily autoxidizes like dihydroxphenylalanine (dopa), probably forming an internally cyclized colored product; therefore, oxidation probably eliminates the α-amino group.[10] The yellowing of 3-amino-L-tyrosine solutions with age is evidence of oxidation.

In the present study, chronologic age of erythrocytes (expressed in days after collection) was not necessarily related to oxidative physiologic aging (Tables 5 and 6). The data (Tables 5 and 6) support the concept that 3-amino-L-tyrosine thermoprotects via an anticatalytic antioxidative mechanism and that the cumulative effects of RBC oxidation can be determined by use of the lu-BSA binding assay. The RBC preincubated in 3-amino-L-tyrosine alone for 72 hours had significantly greater hemolysis than RBC treated with pyruvate or with pyruvate and 3-amino-L-tyrosine. These results and the CL data indicate that hemolysis or thermal fragility taken as a point reading is not a good indicator of physiologic aging or of previous oxidative injury. Furthermore, hemolytic data do not have prognostic value in determining whether or not the cells will be prematurely removed from circulation because of oxidative membrane injury.[17] The 3-amino-L-tyrosine data indicate that the base-line fragility of stored cells is not just a function of oxidative mechanisms, although such mechanisms do contribute to stability of the cell membrane. If 3-amino-L-tyrosine were to be used in long-term storage of RBC, then it would have to be used in conjunction with a carbohydrate energy source

that maintains the adenosine triphosphate concentration of the cells (perhaps inosine in the case of pig cells).

The lu-BSA binding assay has certain advantages over hemolysis for detecting oxidative physiologic aging of RBC. The assay provides prognostic information because it can be used in vitro to determine the sensitivity of RBC to autoxidizable drugs and metabolites. The assay provides rapid diagnostic information because it can measure cumulative peroxidative injury without the need for elaborate procedures.

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